FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)
112843-030	09/936489

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/01795	2 March 2000	11 March 1999

TITLE OF INVENTION

LACTOBACILLUS STRAINS PREVENTING DIARRHOEA PATHOGENIC BACTERIA

APPLICANT(S) FOR DO/EO/US

Neeser et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Return Receipt Postcard.

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/936489	PCT/EP00/01795	112843-030

21. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,000.00	
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO $860.00	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00	
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00	
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	22 - 20 =	2	x $18.00	**$36.00**	
Independent claims	7 - 3 =	4	x $78.00	**$312.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
	TOTAL OF ABOVE CALCULATIONS =			**$1,208.00**	
	Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**. ☐			**$0.00**	
	SUBTOTAL =			**$1,208.00**	
	Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +			**$0.00**	
	TOTAL NATIONAL FEE =			**$1,208.00**	
	Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐			**$0.00**	
	TOTAL FEES ENCLOSED =			**$1,208.00**	
				Amount to be: refunded	$
				charged	$

☒ A check in the amount of **$1,208.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **02-1818** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Robert M. Barrett, Esq.
Bell, Boyd & Lloyd LLC
P.O. Box 1135
Chicago, Illinois 60690-1135
Tel: (312) 807-4204

SIGNATURE

Robert M. Barrett
NAME

30,142
REGISTRATION NUMBER

September 10, 2001
DATE

CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10) Applicant(s): Neeser et al.			Docket No. 112843-030
Serial No. Unknown	Filing Date Herewith	Examiner Unknown	Group Art Unit Unknown

Invention: **LACTOBACILLUS STRAINS PREVENTING DIARRHOEA PATHOGENIC BACTERIA**

I hereby certify that the following correspondence:

Transmittal Letter (duplicate); International Application as Published; Declaration and Power of Attorney (4 pages); Search Report; Preliminary Examination Report; Preliminary Amendment (5 pages); IDS (2 pages); 1449 Form (2 pages); Cited References; Check in the amount of $1208.00; and Return Receipt Postcards.

(Identify type of correspondence)

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231 on

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Neeser et al.
Appl. No.:	PCT/EP00/01795
Filed:	March 2, 2000
Title:	LACTOBACILLUS STRAINS PREVENTING DIARRHOEA PATHOGENIC BACTERIA
Art Unit:	Unknown
Examiner:	Unknown
Docket No.:	112843-030

Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-identified patent application as follows:

In the Claims:

Please amend Claims 3-4 and 7-13 as follows:

3. (Once Amended) The Lactobacillus strain according to claim 1, which is capable of growing in the presence of up to 0.4 % bile salts.

4. (Once Amended) The Lactobacillus strain according to claim 1, which is selected from the group consisting of Lactobacillus rhamnosus and Lactobacillus paracasei.

7. (Once Amended) A method for preparing an ingestable support material comprising using a lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea.

8. (Once Amended) The method according to claim 7, wherein the Lactobacillus strain is contained in the ingestable support material in an amount from about 10^5 cfu / g to about 10^{12} cfu / g support material.

9. (Once Amended) A method for preparing an ingestable support comprising the steps of using a supernatant of a culture of a lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea.

10. (Once Amended) The method of claim 9, wherein the ingestable support is a food composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, and infant formulae.

11. (Once Amended) A method for the treatment of a disorder associated with diarrhoea comprising the step of administering a lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea.

12. (Once Amended) A pharmaceutical composition containing at least one lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea or a supernatant of a culture thereof.

13. (Once Amended) The method according to claim 11 wherein the lactic acid bacterium strain is in a composition which is selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, tablets, liquid bacterial suspensions, dried oral supplement, liquid oral supplement, dry tube feeding and liquid tubefeeding.

Please add newly submitted claims 14-22 as follows:

14. The pharmaceutical composition according to claim 12 wherein the Lactobacillus strain is capable of adhering to the intestinal mucosa of a host organism.

15. The pharmaceutical composition according to claim 12 wherein the Lactobacillus strain is capable to grow in the presence of up to 0.4 % bile salts.

16. The pharmaceutical composition according to claim 12 wherein the Lactobacillus strain is selected from the group consisting of Lactobacillus rhamnosus and Lactobacillus paracasei.

17. The pharmaceutical composition according to claim 12 wherein the Lactobacillus strain is Lactobacillus paracasei.

18. The pharmaceutical composition according to claim 12 wherein the Lactobacillus strain is Lactobacillus paracasei CNCM I-2116 (NCC 2461).

19. The method of claim 7 wherein the ingestable support material is a food composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, and infant formula.

20. A method for preventing a disorder associated with diarrhea comprising the step of administering to a patient at risk of such disorder a lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhea.

21. A food containing a lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea.

22. The food according to claim 21 wherein the food is selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, tablets, liquid bacterial suspensions, dried oral supplement, liquid oral supplement, dry tube feeding and liquid tubefeeding.

REMARKS

This Preliminary Amendment is submitted in the above-identified patent application. Pursuant to the Preliminary Amendment Claims 3-12 have been amended and newly submitted Claims 20-22 have been added. This Preliminary Amendment does not add new matter. Further, Applicants note that the Preliminary Amendment is not being made to narrow the claims and/or for purposes of patentability but, merely to comport the claims to U.S. practice and/or to add additional claims. Applicants therefore do not intend to disclaim any subject matter in view of the amendments.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**<u>Versions with Markings to Show Changes Made.</u>**"

Respectfully submitted,

BELL, BOYD & LLOYD LLC

BY

Robert M. Barrett
Reg. No. 30,142
P.O. Box 1135
Chicago, Illinois 60690-1135
Phone: (312) 807-4204

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

Claims 3-4 and 7-13 have been amended as follows:

3. (Once Amended) The Lactobacillus strain according to [any of the preceding claims] claim 1, which is capable [to grow] of growing in the presence of up to 0.4 % bile salts.

4. (Once Amended) The Lactobacillus strain according to [any of the preceding claims] claim 1, which is selected from the group consisting of Lactobacillus rhamnosus [or] and Lactobacillus paracasei.

7. (Once Amended) A method for preparing [Use of a Lactobacillus strain according to any of the preceding claims for the preparation of] an ingestable support material comprising using a lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea.

8. (Once Amended) The method [use] according to claim 7, wherein the Lactobacillus strain is contained in the ingestable support material in an amount from about 10^5 cfu / g to about 10^{12} cfu / g support material.

9. (Once Amended) A method for preparing an ingestable support comprising the steps of using [Use of] a supernatant of a culture of a lactic acid bacterium strain belonging to the genus Lactobacillus [strain according to any of the claims 1 to 6 for the preparation of an ingestible support] having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea.

10. (Once Amended) The method of [use according to any of the claims 7 or] claim 9, wherein the ingestable support [material] is a food composition selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, and infant formulae.

11. (Once Amended) A method [The use according to any of the claims 7 to 10, wherein the support material is used] for the treatment [and/or prophylaxis] of a disorder [disorders] associated with diarrhoea comprising the step of administering a lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea.

12. (Once Amended) A [Food or] pharmaceutical composition containing at least one lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea [Lactobacillus strain according to any of the claims 1 to 6] or a supernatant of a culture thereof.

13. (Once Amended) The [composition] method according to claim [12,] 11 wherein the lactic acid bacterium strain is in a composition which is selected from the group consisting of milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, tablets, liquid

bacterial suspensions, dried oral supplement, [wet] liquid oral supplement, dry tube feeding [or wet] and liquid tubefeeding.

Claims 14-22 have been added.

LACTOBACILLUS STRAINS PREVENTING DIARRHOEA PATHOGENIC BACTERIA

The present invention pertains to novel microorganisms of the genus Lactobacillus, that are useful in preventing diarrhoea brought about by pathogenic bacteria. In particular, the present invention relates to the use of said microorganisms for the preparation of an ingestable support and to a composition containing the same.

Organisms that produce lactic acid as a major metabolic component have been known for a long time. These bacteria may be found in milk or in milk processing factories, respectively, living or decaying plants but also in the intestine of man and animals. These microorganisms, summarized under the term "lactic acid bacteria", represent a rather inhomogeneous group and comprise e.g. the genera Lactococcus, Lactobacillus, Streptococcus, Bifidobacterium, Pediococcus etc..

Lactic acid bacteria have been utilized as fermenting agents for the preservation of food taking benefit of a low pH and the action of fermentation products generated during the fermentative activity thereof to inhibit the growth of spoilage bacteria. To this end, lactic acid bacteria have been used for preparing a variety of different foodstuff such as cheese, yogurt and other fermented diary products from milk.

Quite recently lactic acid bacteria have attracted a great deal of attention in that some strains have been found to exhibit valuable properties to man and animals upon ingestion. In particular, specific strains of the genus Lactobacillus or Bifidobacterium have been found to be able to colonize the intestinal mucosa and to assist in the maintenance of the well-being of man and animal.

In this respect, EP 0 768 375 discloses specific strains of the genus Bifidobacterium, that are capable to become implanted in the intestinal flora and may adhere to intestinal cells. These Bifidobacteria are reported to assist in immunomodulation,

being capable to competitively exclude adhesion of pathogenic bacteria to intestinal cells, thus assisting in the maintenance of the individual's health.

During the last few years research has also focused on the potential use of lactic acid bacteria as probiotic agents. Probiotics are considered to be viable microbial preparations which promote the individual's health by preserving the natural microflora in the intestine. A microbial preparation may be commonly accepted as a probiotic in case the effectual microbes thereof and their mode of action are known. Probiotics are deemed to attach to the intestine's mucosa, colonize the intestinal tract and likewise prevent attachment of harmful microorganisms thereon. A crucial prerequisite for their action resides in that they have to reach the gut's mucosa in a proper and viable form and do not get destroyed in the upper part of the gastrointestinal tract, especially by the influence of the low pH prevailing in the stomach.

In this respect, WO 97/00078 discloses a specific strain, termed Lactobacillus GG (ATCC 53103), as such a probiotic. The microorganism is particularly employed in a method of preventing or treating food induced hypersensitivity reactions in that it is administered to a recipient together with a food material that has been subjected to a hydrolysis treatment with pepsin and/or trypsin. The Lactobacillus strain selected is described as exhibiting adhesive and colonizing properties and showing a protease enzyme system, so that the protein material contained in the foodstuff to be administered is further hydrolyzed by means of proteases secreted by the specific Lactobacillus strain. The method discussed in this document shall eventually result in the uptake of protein material by the gut that does not show a substantial amount of allergenic material anymore.

Further, in EP 0 577 903 reference is made to the use of such lactic acid bacteria having the ability of replacing Heliobacter pylori, the acknowledged cause for the

development of ulcer, in the preparation of a support intended for the therapeutic or prophylactic treatment of an ulcer associated with the action of Heliobacter pylori.

In knowledge of the valuable properties particular strains of lactic acid bacteria may provide, there is a desire in the art for additional lactic acid bacterial strains that are beneficial to the well being of man and/or animal.

Consequently, a problem of the present invention is to provide additional bacterial strains that exhibit new properties beneficial for man and/or animals.

The above problem has been solved by providing novel microorganisms, namely lactic acid bacteria, belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea.

According to a preferred embodiment the Lactobacillus strain is capable of adhering to the intestinal mucosa of mammal and may grow in the presence of up to 0.4 % bile salts.

Yet, according to another preferred embodiment the lactic acid bacterium is selected from the group consisting of Lactobacillus rhamnosus or Lactobacillus paracasei, preferably Lactobacillus paracasei, and is more preferably Lactobacillus paracasei CNCM I-2116.

The microorganisms of the present invention have been shown to exhibit inter alia the following properties: they are gram positive, catalase negative, NH_3 form arginine negative and CO_2 production negative, they produce L(+) lactic acid, are capable to grow in the presence of bile salts in a concentration of up to about 0.4 % and may essentially prevent colonization of intestinal cells by bacteria bringing about diarrhoea, such as pathogenic E. coli, e.g. enteropathogenic E.coli (EPEC), or salmonella, e.g. salmonella typhimurium.

The novel microorganisms may be used for the preparation of a variety of ingestable support materials, such as e.g. milk, yogurt, curd, fermented milks, milk based fermented products, fermented cereal based products, milk based powders, infant formulae and may be included in the support in an amount of from about 10^5 cfu / g to about 10^{11} cfu / g. For the purpose of the present invention the abbreviation cfu shall designate a "colony forming unit" that is defined as number of bacterial cells as revealed by microbiological counts on agar plates.

The present invention also provides a food or a pharmaceutical composition containing at least one of the Lactobacillus strains having the above traits and/or containing a supernatant of a culture, in which the microorganisms have been grown or a fraction thereof, respectively.

For preparing a food composition according to the present invention at least one of the Lactobacillus strains of the present invention is incorporated in a suitable support, in an amount of from about 10^5 cfu / g to about 10^{11} cfu / g, preferably from about 10^6 cfu / g to about 10^{10} cfu / g, more preferably from about 10^7 cfu / g to about 10^9 cfu / g.

In case of a pharmaceutical preparation the product may be prepared in forms of tablets, liquid bacterial suspensions, dried oral supplements, wet oral supplements, dry tube feeding or a wet tube feeding with the amount of Lactobacillus strains to be incorporated therein being in the range of up to about 10^{12} cfu / g, preferably from about 10^7 cfu / g to about 10^{11} cfu / g, more preferably from about 10^7 cfu / g to about 10^{10} cfu / g.

The activity of the novel microorganisms in the individual's intestine is naturally dose dependent. That is, the more the novel microorganisms are incorporated by means of ingesting the above food material or the pharmaceutical composition the higher the

protective and/or curing activity of the microorganisms. Since the novel microorganisms are not detrimental to mankind and animals and have eventually been isolated from baby feces a high amount thereof may be incorporated so that essentially a high proportion of the individual's intestine will be colonized by the novel microorganisms.

Yet, according to another preferred embodiment the supernatant of a culture of a Lactobacillus strain of the present invention may be used for preparing one of the above ingestable support. The supernatant may be used as such or may well be dried under conditions that do not destroy the metabolic compounds secreted by the microrganisms into the liquid medium, such as e.g. freeze drying, and may be included in the carrier. In order to minimize the number of unknown compounds in the supernatant the Lactobacilli strains will preferably be grown in a defined media, the composition of which is known and does not negatively affect the host incorporating it. Further, the skilled person will, based on his general knowledge optionally deplete the supernatant from unwanted products, such as e.g. by means of chromatography.

In the figures,

Fig. 1 shows a scheme illustrating the results of a cell culture experiment in which cultured ST11 cells were used in an assay for inhibiting adhesion of pathogenic E. coli bacteria to epithelial cells.

Fig. 2 shows a scheme illustrating the results of a cell culture experiment, in which the supernatant of a ST11 culture was used in an assay for inhibiting adhesion of pathogenic E. coli bacteria to epithelial cells.

Fig. 3 shows a scheme illustrating the results of a cell culture experiment, in which cultured ST11 cells were used in an assay for inhibiting invasion of salmonella typhimurium into epithelial cells.

Fig. 4 shows a scheme illustrating the results of a cell culture experiment, in which the supernatant of a ST11 culture was used in an assay for inhibiting invasion of salmonella typhimurium into epithelial cells.

Fig. 5 shows the acidification of the L. casei strain CNCM I-2116 (termed ST11) in different growth media.

Fig. 6 shows the survival rate of the L. casei strain ST11 at 10 °C measured during 30 days.

Fig. 7 shows the mRNA pattern of IL-12 and IL-10 in mouse adherent cells derived from bone marrow after incubation of the cells with serial dilutions of ST11.

Fig. 8 shows the outcome of Th2 differentiation as resulting in decreased IL-4 production.

During the extensive studies leading to the present invention the inventors have investigated baby feces and isolated a variety of different bacterial strains therefrom. These strains were subsequently examined for their capability to prevent colonization of epithelial cells with bacteria that are known to cause diarrhoea.

Several bacterial genera comprising Lactobacillus, Lactococcus and Streptococcus were screened for their diarrhoea inhibitory properties. The tests for the inhibitory property were essentially performed with pathogenic E. coli and salmonella typhimurium as representative for pathogenic microorganisms causing diarrhoea in an affected individual.

The various lactic acid bacteria were grown in a suitable medium, such as MRS, Hugo-Jago or M17 medium at temperatures of from about 30 to 40°C corresponding to their optimal growth temperature. After reaching stationary growth the bacteria were collected by centrifugation and resuspended in physiological NaCl solution. Between the different tests the bacterial cells were stored frozen (-20°C).

For assessing anti-bacterial properties the following approaches were chosen.

According to one protocol cultured Lactobacillus strains of the present invention were examined for their capability to prevent adhesion of pathogenic bacteria causing diarrhoea to intestinal cells or invasion thereof into intestinal cells, respectively. To this end, intestinal cells were contacted with the pathogenic bacteria and the cultured Lactobacillus strains of the present invention, and the rate of adhesion, or invasion, respectively, was assessed.

According to a second protocol the supernatant of a cell culture of the Lactobacillus strains of the present invention was added together with the pathogenic micro-organisms to the intestinal cells and the rate of adhesion, or invasion, respectively, was assessed.

Thus, it could be shown that the cultured Lactobacilli and the supernatant proofed to be extremely effective to prevent both adhesion to and invasion into the intestinal cells indicating that metabolic compounds secreted by the novel microorganisms are likely to be responsible for the anti-diarrhoea activity.

In addition to the above finding it could also be shown that the strains of the present invention surprisingly also exhibit anti-allergenic properties in that said strains have an impact on the synthesis of different immunological mediators.

It is generally acknowledged that humoral immune responses and allergic reactions are mediated by $CD4^+$ T cells bearing the type 2 phenotype (Th2). Th2-cells are characterized by the production of high levels of interleukin 4 (IL-4), a cytokine required for the secretion of IgE, which is the major antibody class involved in allergic reactions.

The differentiation of Th2 cells is impaired by IFN-γ, a particular cytokine that is produced by the mutually exclusive Th1 subset of $CD4^+$ T cells. Said Th1 cells are in turn strongly induced by interleukin 12 (IL-12). In contrast thereto IL-10, another cytokine, has been shown to have a strong suppressing impact on the proliferation of Th1 cells and is therefore deemed to play a role in immuno-supressive mechanisms.

In summary, both IL-12 and IL-10 have strong modulatory effects on $CD4^+$ T cell development by influencing the development of the Th1 subset. IL-12 is a key regulatory cytokine for the induction of Th1 differentiation and thus inhibits the generation of Th2 responses. A major pathway for inhibition of Th2 cells is therefore seen in the stimulation of IL-12 synthesis by accessory cells.

It is well known that some components of gram negative bacteria, such as LPS, induce high levels of IL-12 in adherent cells, such as macrophages and dendritic cells. Consistently, it has been found that gram negative bacteria can strongly bias $CD4^+$ T cell differentiation towards the Th1 phenotype.

The microorganism ST11 as an example of the Lactobacillus strains of the present invention has been tested for a potential role in the induction of cytokines involved in the regulation of $CD4^+$ T cell differentiation. In particular, the effect of ST11 on the phenotype of $CD4^+$ T cells undergoing Th2 differentiation has been studied.

In this respect the capacity of ST11 to induce the synthesis of mRNA encoding these two regulatory cytokines in mouse adherent cells derived from bone marrow was

compared with 4 other strains of Lactobacilli and with a control of gram negative bacteria (E. coli K12). The mRNA was measured by semi-quantitative RT-PCR after 6 hours of incubation of the cells with serial dilutions of bacteria ranging from 10^7 to 10^9 cfu/ml.

Although all strains of Lactobacillus could induce transcription of IL-12 mRNA to a certain degree, ST11 could be shown to be the strongest inducer, since as a strong PCR signal could be detected even at the lowest bacterial dose. In fact, the capacity of ST11 to induce IL-12 mRNA transcription was as strong as that of E. coli. Induction of IL-10 mRNA was in general weaker than for IL-12 mRNA, as only at higher bacterial doses a signal could be detected. Nevertheless, ST11 was the strongest inducer of IL-10 mRNA, as compared to the other Lactobactilli and the E.coli control.

Thus, ST11 is deemed to be efficient in inducing immunoregulatory cytokines involved in $CD4^+$ T cell differentiation. Its strong capacity to induce IL-12 makes it a candidate to inhibit Th2 responses and its measurable IL-10 induction may prevent inflammatory responses.

In addition to the above finding it was also determined whether ST11 had an inhibitory effect on $CD4^+$ T cells undergoing Th2 differentiation and a positive effect on Th1 functions. A well established cell differentiation culture system was utilized, where precursor $CD4^+$ T cells were polyclonally activated and modulated to undergo either Th1 or Th2 differentiation, depending on the type of co-stimuli provided in the culture medium. Th1/Th2 differentiation was induced during a 7-days primary culture, after which the cells were then restimulated for 2 days in a secondary culture containing medium alone and acquisition of a specific phenotype (Th1 or Th2) was assessed by measuring the types of cytokines produced in the supernatant (IFN-γ vs. IL-4).

It is generally known that precursor $CD4^+$ T cells from mice of the BALB/c background preferentially differentiate to predominant Th2 phenotype (high IL-4, low IFN-γ in the 2ry culture supernatants) after activation under neutral conditions (medium alone in the 1ry culture). This phenotype could be completely reverted to a Th1 pattern (high IFN-γ, low IL-4) upon addition of a blocking monoclonal antibody to IL-4 in the 1ry culture.

To investigate a potential role for ST11 on Th2 inhibition, purified precursor $CD4^+$ T cells from BALB/c mice were activated in the presence of bone marrow adherent cells as accessory cells during the 1ry culture. These cells were co-cultured either in medium alone, or in the presence of 1 mg/ml LPS, or 10^8 cfu/ml ST11, or 10^8 cfu/ml of another Lactobacillus. After this time, the cells were washed and $CD4^+$ T cells were purified once again and restimulated in the 2ry culture in medium alone.

Cytokines produced by the differentiated $CD4^+$ T cells were measured after 2 days. As expected, cells that differentiated in the presence of medium alone displayed a dominant Th2 phenotype. Addition of ST11 to the 1ry cultures strongly modulated the outcome of Th2 differentiation, as it resulted in an 8-fold decrease in IL-4 production. This inhibition was of similar magnitude as that observed in cultures derived from cells differentiated in the presence of LPS. In contrast thereto, the other Lactobacillus strain had no measurable impact on IL-4 levels. Interestingly, IFN-γ levels were not increased upon addition of ST11 in the 1ry cultures.

In summary, ST11 specifically impaired IL-4 production by $CD4^+$ T cells undergoing Th2 differentiation, but did not significantly increase IFN-γ secretion. The fact that ST11 does not increase IFN-γ production may be due to its capacity to induce IL-10 with the consequence that it may keep a low inflammatory impact despite its anti-Th2 activity.

In consequence, it could be shown that ST11 is one of Lactobacillus strains that have a good anti-Th2 profile which makes them excellent candidates for their use as a bacterium with anti-allergic, probiotic activity.

The present invention will now be described by way of examples without limiting the same thereto.

Media and solutions:

MRS (Difco)

Hugo-Jago (tryptone 30g / l (Difco), yeast extract 10 g / l (Difco), lactose 5 g / l (Difco), KH_2PO_4 6 g / l, beef extract 2 g / l (Difco), agar 2 g / l (Difco))

M17 (Difco)

DMEM (Dulbecco's modified Eagle medium)

CFA (according to Ghosh et al. Journal of Clinical Microbiology, 1993 31 2163-6)

Müller Hinton agar (Oxoid)

LB (Luria Bertami, Maniatis, A Laboratory Handbook, Cold Spring Harbor, 1992)

Antibiotics were obtained from Sigma

C^{14}-acetate (53,4 Ci/mMol, Amersham International PLC)

PBS (NaCl 8g/l, KCl 0.2 g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.2 g/l))

Trypsin-EDTA solution (Seromed)

FCS Fetal calf serum (Gibco)

E. coli DAEC C 1845 was obtained from Washington University, Seattle and E. coli JPN15 was obtained from the Center for Vaccine Development of the University of Maryland, USA). The salmonella typhimurium strain SL1344 was obtained from the department of microbiology, Stanford University, CA, USA).

Example 1

Isolation of lactic acid bacteria from baby feces

Fresh feces were harvested from diapers of 16 healthy babies 15 to 27 days old. 1 g of fresh feces was placed under anaerobic conditions for transportation to the laboratory and microbiological analyses were run within 2 hours from sampling by serial dilutions in Ringer solution and plating on selective media. MRS agar plus antibiotics (phosphomycine 80 µg/ml, sulfamethoxazole 93 µg/ml, trimethoprime 5µg/ml) incubated at 37°C for 48 hours was used to isolate lactic acid bacteria. Colonies were randomly picked up and purified. Physiological and genetic characterization was performed on the isolates.

Example 2

Cultivating Caco-2 cells

For the inhibition assays the cell line Caco-2 was utilized as a model of the intestine. This cell line presents features characteristic for intestinal cells such as e.g. polarisation, expression of intestinal enzymes, production of particular structural polypeptides etc..

The cells were grown on three different supports, namely on plastic dishes (25 cm^2, Corning) for growth and propagation, on defatted and sterilized 6 well glass plates (22 x 22 mm, Corning) for the adhesion tests, and on 24 well glass plates (Corning) for the inhibition tests.

After the second day in culture the medium (DMEM) was changed on a daily basis. Before use the medium was supplemented with 100 U/ml penicilline / streptomycine, 1 µg/ml amphoterine and 20 % FCS inactivated at 56 ° for 30 min. Culturing was performed at 37 °C in an atmosphere comprising 90% air and 10% CO_2. The cells were splitted every six days. The cells were detached from the walls of the well by treatment in PBS with 0.25 % trypsin and 3 mM EDTA at pH 7.2. For neutralizing

the effect of trypsin an equal volume of FCS was added to the cell suspension obtained, the mixture was centrifuged (10 min at 1000 rpm) and the pellet was again put in culture. About 3.5 x 10^5 cells were transferred to a new culture bottle and cultivated until a confluent monolayer was obtained.

Example 3

Cultivating bacteria

ST11:

The bacterial strain has been stored at -20 °C in MRS medium containing 15 % glycerol. The strain has been grown under anaerobic conditions in MRS and transferred twice to new media at intervals of 24 hours before use in the inhibition assays. For the assay a concentration of 2 x 10^9 cfu/ml was utilized.

The supernatant was collected by centrifugation for 1 hour at 20.000 rpm and the supernatant obtained was subsequently checked for the presence of bacteria.

E. coli:

Two E. coli strains were used, E. coli DAEC C 1845 (Diffuse Adhesion E. Coli) and E. coli JPN15 (EPEC; Entero-Pathogenic E. Coli).

The first passage after thawing was effected on a CFA - Müller Hinton agar, which is suitable to effect expression of adhesion factors by the bacterium.

Before each experiment the bacterial cells were incubated at 37 °C with a transfer to a new medium being effected twice after 24 hours each. Since JPN15 contains the gene for ampicilline resistance said antibiotic was used for selection during growth.

Salmonella:

The salmonella typhimurium strain SL1344 was utilized for the experiments, which was grown before usage in LB medium.

Example 4

Inhibition assay for E. coli

After the second passage to new medium the pathogenic bacterial strains were marked with radioisotopes using C^{14}-acetat at 10 μCi/ml in LB-medium. Incubation of the strains in this medium was performed for 18 hours at 37 °C.

The bacterial suspension was subsequently subjected to centrifugation (1041 g, 15 min) so as to eliminate the supernatant with the remaining C^{14}-acetate. The pellet was suspended and washed in PBS and the cells were suspended at a concentration of about 10^8 cells / ml in 1 % sterile mannose. The mannose is known to inhibit non specific adhesion.

The different pathogenic bacterial strains (E. coli) were contacted with a monolayer of the Caco-2 cells (37 °C, 10 % CO_2, 90 % air) for 3 hours. The same experiments were carried out using supernatant (obtained by centrifuging at 20.000 rpm for 40 min).

As a control the pathogenic bacteria were contacted with the Caco-2 monolayer without concurrent addition of ST11 or a culture supernatant, respectively.

After 3 hours incubation the medium was changed and the monolayer was washed three times with PBS. Each washing step included 20 x stirring of the PBS solution so as to eliminate essentially all of non specific adhesion. The cells were lysed thereafter by addition of 1 ml sodium carbonate and incubation for 40 min at 37 °C. After homogenization an aliquot (250 μl) was diluted in 5 ml scintillation fluid (Hionic-

fluor Packcard) and counted (Packard 2000). The percentage of the adhesion of pathogenic cells to Caco-2 cells was calculated against the control, which was set to 100 % (adhesion; or invasion for example 5).

Example 5

Inhibition assay for salmonella

Salmonella are bacteria that invade epithelial cells and multiply therein. For determining the inhibitory activity of ST11 salmonella typhimurium strain SL1344 was incubated as described above in a medium containing ^{14}C-acetate and was subjected to the experiment described in example 4.

After incubation the Caco-2 cells were washed with PBS so as to eliminate all non-adhering cells. Subsequently medium was added containing gentamycin (20 μg/ml) and incubation was continued for 1 hour at 37 °C. Gentamycin is an antibiotic not penetrating intestinal cells so that all extracellular microorganisms were killed, while salmonella having already invaded intestinal cells will survive. After washing the cells twice with PBS the cells were lysed by addition of sterile distilled water and the radioactivity was measured as described in example 4.

The results of experiments 4 and 5 are shown in figures 1 to 4. It may be seen that cultured ST11 cells and the culture supernatant were extremely effective in preventing adhesion of and invasion into intestinal cells by pathogenic microorganisms causing diarrhoea.

Example 6

Properties of ST11

ST 11 has been subjected to incubation in simulated gastric juice. The simulated gastric juice was prepared by suspending pepsin (3 g/l) in sterile saline (0.5% w/v)

and adjusting the pH to 2.0 and 3,0, respectively, with concentrated HCl. ST 11 has been grown in varying amounts in the above media and the resistance of the microorganisms has been determined.

The results are summarized in table I below .

Table I

pH	Cfu/ml at T 0	Cfu at T 1min	Cfu at T 15	cfu at T 30	cfu at T 60
2.0	2.0×10^9	1.8×10^9	1.2×10^9	3.7×10^8	7.0×10^3
3.0	2.0×10^9	1.9×10^9	1.7×10^9	1.7×10^9	8.4×10^8

ST11 has the following properties as defined according to methods disclosed in the genera of lactic acid bacteria, Ed. B.J.B. Wood and W.H. Holzapfel, Blackie A&P.

- gram positive,
- catalase negative,
- NH_3 form arginine negative
- CO_2 production negative
- production of L(+) lactic acid
- growth in the presence of bile salts in a concentration of up to about 0.4 %.

Example 7

Growth of ST 11 under different conditions

ST11 was incubated at 37 °C in tomato based medium (4% tomato powder rehydrated in distilled water) supplemented with sucrose (0, 0.5, 1 or 2%) or soya peptone (0.5%) or glucose (0.5%) for different periods of time. The results are shown in fig. 5.

ST11 was further added in an amount of 2.5 % to a medium composed of rice flour (3%), wheat flour (2%) and sucrose (3%) and incubated at 37°C until a pH of 4.4

was reached. After cooling the product was packed with or without addition of vitamine C and stored at 10°C.

Fig. 5 shows survival data of ST 11 at 10°C in a cereal drink packed in different plastic materials (HDPE High density polyethylene, PS polystyrene).

Example 8

Induction of IL-12 and IL10- mRNA synthesis in mouse adherent cells by ST11

Bone marrow cells were isolated from the femur and tibia of 8 week-old specific pathogen-free C57BL/6 mice and were incubated at a concentration of $2x10^6$ cells/ml in RPMI medium (Gibco) containing 10% fetal bovine serum, 1 mM L-Glutamine, 12 mM Hepes, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin (all reagents from Gibco) for 12 hours at 37°C in a 5% CO_2 atmosphere. Non-adherent cells were discarded by 3 consecutive washes with warm culture medium and the remaining adherent cells were harvested and incubated at a concentration of 10^6 cells/ml for 6 hours in the presence or absence of bacteria. It was previously determined that 6 hours represents an optimal time-point for cytokine mRNA synthesis by mouse adherent cells in response to LPS. Bacteria were added at different concentrations ranging from 10^9 to 10^7 cfu/ml. Bacteria were grown and stored as indicated above (page 6).

At the end of the 6-hour culture period, cells were isolated by centrifugation and lysed using the TRIzol reagent kit (GibcoBRL, Cat. No. 15596-018) following the manufacturer's instructions. Total RNA was isolated by isopropanol precipitation and reverse-transcribed into cDNA for 90 min at 42°C using 200 U reverse transcriptase (Superscript II, BRL) in a 40-µl reaction volume containing 200 mM Tris pH 8.3, 25 mM KCl, 1 µg/ml oligo $d(T)_{15}$ (Boehringer Mannheim), 1 mM DTT (Boehringer Mannheim), 4 mM of each dNTP (Boehringer Mannheim) and 40 U/ml Rnasin (Promega). PCR primers and conditions were used as already described in Kopf et al.

(Journal of Experimental Medicine 1996 Sep 1;184(3):1127-36). Amounts of cDNA were normalized within the samples using primers specific for a house-keeping gene (β-2-microglobulin). PCR products were separated on a 2% agarose gel and bands were analyzed under UV.

As shown in Figure 7, ST11 showed the strongest induction of IL-12 and IL-10 mRNA, which was comparable to levels observed with the positive control (E. coli). Differences are best seen at the lowest bacteria concentrations (10^7 cfu/ml).

Example 9

Suppression of IL-4 synthesis by ST11

$CD4^+$ T cells were purified from the spleen of specific pathogen-free BALB/c mice using the MiniMACS kit from Miltenyi Biotec (Cat. No. 492-01). The $CD4^+$ T cells were cultured at a concentration of $2x10^5$ cells/ml in RPMI medium containing 10% fetal bovine serum, 1 mM L-Glutamine, 12 mM Hepes, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin and activated during one week by cross-linking with plate-bound monoclonal antibodies to CD3 (clone 2C11) and CD28 (clone 37.51, both antibodies from Pharmingen). During this 1ry culture, the $CD4^+$ T cells were co-cultured with bone marrow adherent cells (isolated as described above) as accessory cells and with 10^8 cfu/ml ST11, or 10^8 cfu/ml La1, or 1 mg/ml LPS, or medium alone. After this time, the cells were washed and $CD4^+$ T cells were purified once again using MiniMACS kit technology and restimulated in a 2ry culture containing medium alone. Cytokines produced by the differentiated $CD4^+$ T cells were measured in the supernatants after 2 days using sandwich ELISA (kits from Endogen and Pharmingen).

Results are shown in Fig. 5. Cells differentiated in the presence of medium alone displayed a dominant Th2 phenotype characterized by high levels of IL-4. Addition of ST11 to the 1ry cultures strongly modulated the outcome of Th2 differentiation, as it

resulted in an 8-fold decrease in IL-4 production. This inhibition was of similar magnitude as that observed in cultures derived from cells differentiated in the presence of LPS. In contrast thereto, the other Lactobacillus strain had no measurable impact on IL-4 levels. Interestingly, IFN-γ levels were not increased upon addition of ST11 in the 1ry cultures.

As may be seen from the above the strains of the present invention may be well prepared for the production of a food and/or a pharmaceutical carrier taking advantage of the valuable properties of the microorganisms.

Example 10

The ST11 strain was tested in a clinical trial in a community on the outskirts of Guatemala City on its ability to influence the transmission and experience of acute rainy-season diarrhoeal disease experienced by most of the children in this area. A total of 203 children, aged 35 to 70 months were enrolled in the study and received a target dose of 10^{10} viable organisms (ST11) or none (placebo) over a feeding period of 29 days. The children selected for both sample and placebo, respectively, had the typical deficits in weight-for-age and height-for-age characteristic due to undernutrition.

Before initiating the feeding trial in preschool children, a safety evaluation was conducted based on *in vitro* and *in vivo* studies. In vitro studies showed antibiotic resistance pattern similar to those of other lactobacilli used in food applications, and no potential for forming biogenic amines, for degrading mucin and for deconjugating bile salts. In a placebo-controlled clinical study involving 42 adult volunteers, ST11 was well tolerated and did not induce any adverse effects, among the potential manifestations monitored, such as flatulence, number of stools per day and stool consistency; the levels of acute-phase proteins in serum did not raise any concern with respect to a potential inflammatory reaction.

The samples and placebo's have been packed in sachets at the Nestlé Product Technology Center manufacturing facility in Konolfingen, Switzerland, and shipped refrigerated to Guatemala. Each 10 g sachet consisted of a chocolate flavored vehicle and either 0.2 g of ST11 (10^{10} cfu) or, in case of the placebo, 0.2 g milk powder. The chocolate flavored vehicle consisted of cocoa powder, sugar, soy lecithin, vanillin, and cinnamon. The sachets were stored at 4° to 6°C until two hours before use. Before use the sachet had to be dissolved in 100 ml water provided by Nestlé, which was free of any bacterial contamination.

According to the protocol applied diarrhoea was defined as the occurrence of three or more liquid or unformed fecal evacuation during a period of 24 hrs. A diarrhoeal episode was defined as an event that presented the evidence of diarrhoea (3 diarrhoeal evacuations over 24 hrs). Its total duration in hrs was calculated from the moment of the first of the three index stools to the appearance of the first formed stool or a period 24 h without any defecation. For a child to have a "new" episode, 48 hours had to have elapsed the termination of the prior episode. If not, it was considered a continuation of the same episode and the total duration was then used for evaluation. A case was a child who experienced one or more documented episodes of diarrhoea through the 29-day observation period. Intensity of a diarrhoeal episode was based on the total number of loose stools produced. The elements of severity of the episode embraced the presence of blood, mucus or pus in stools, along with symptoms of fever and vomiting. An intensity of 7 stools per 24 h or the need for intervention by a health professional at a clinic, health center or hospital also classified an episode as severe.

When a diarrhoeal episode was diagnosed through the surveillance system, a specimen of a diarrhoeal stool was collected for microscopic examination and culture to identify potential etiological pathogens for that episode. The specimen was diagnosed for rotavirus antigen, Giardia, and E. histolytica, in case the sample was dysenteric, and for bacterial pathogens including Shigella, Salmonella, Aeromonas, Plesiomonas shigelloides, E. Coli and possibly V. cholerae.

During the period of investigation product samples were collected to examine the viability of the microorganisms included during the period of administration. It could be shown that the microorganism stayed viable in the sachets during the entire study so that also at the end of the study the sachets were capable to convey 10^{10} viable microorganism upon reconstitution with water.

The study revealed that the sample containing the probiotic microorganism could decrease the occurrence of diarrhoea in contrast to the control group (placebo) by about 30 %. Yet, also the control group already exhibited a decreased number of diarrhoeal occurrence over the normal population receiving none of the samples or placebo, respectively. This latter finding may in part be explained on the basis of the children receiving additional valuable nutrition and contamination free water. However, since the study was performed in the field it may clearly be derived that ST11 can surely reduce the occurrence of diarrhoea in vivo.

Claims

1. Lactic acid bacterium strain belonging to the genus Lactobacillus having the capability of preventing colonization of the intestine with pathogenic bacteria causing diarrhoea.

2. The Lactobacillus strain according to claim 1, which is capable of adhering to the intestinal mucosa of a host organism.

3. The Lactobacillus strain according to any of the preceding claims, which is capable to grow in the presence of up to 0.4 % bile salts.

4. The Lactobacillus strain according to any of the preceding claims, which is selected from the group consisting of Lactobacillus rhamnosus or Lactobacillus paracasei.

5. The Lactobacillus strain according to claim 4, which is Lactobacillus paracasei.

6. The Lactobacillus paracasei according to claim 5, which is Lactobacillus paracasei CNCM I-2116 (NCC 2461).

7. Use of a Lactobacillus strain according to any of the preceding claims for the preparation of an ingestable support material.

8. The use according to claim 7, wherein the Lactobacillus strain is contained in the support material in an amount from about 10^5 cfu / g to about 10^{12} cfu / g support material.

9. Use of a supernatant of a culture of a Lactobacillus strain according to any of the claims 1 to 6 for the preparation of an ingestible support.

10. The use according to any of the claims 7 or 9, wherein the support material is a food composition selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae.

11. The use according to any of the claims 7 to 10, wherein the support material is used for the treatment and/or prophylaxis of disorders associated with diarrhoea.

12. Food or pharmaceutical composition containing at least one Lactobacillus strain according to any of the claims 1 to 6 or a supernatant of a culture thereof.

13. The composition according to claim 12, which is selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, tablets, liquid bacterial suspensions, dried oral supplement, wet oral supplement, dry tube feeding or wet tubefeeding.

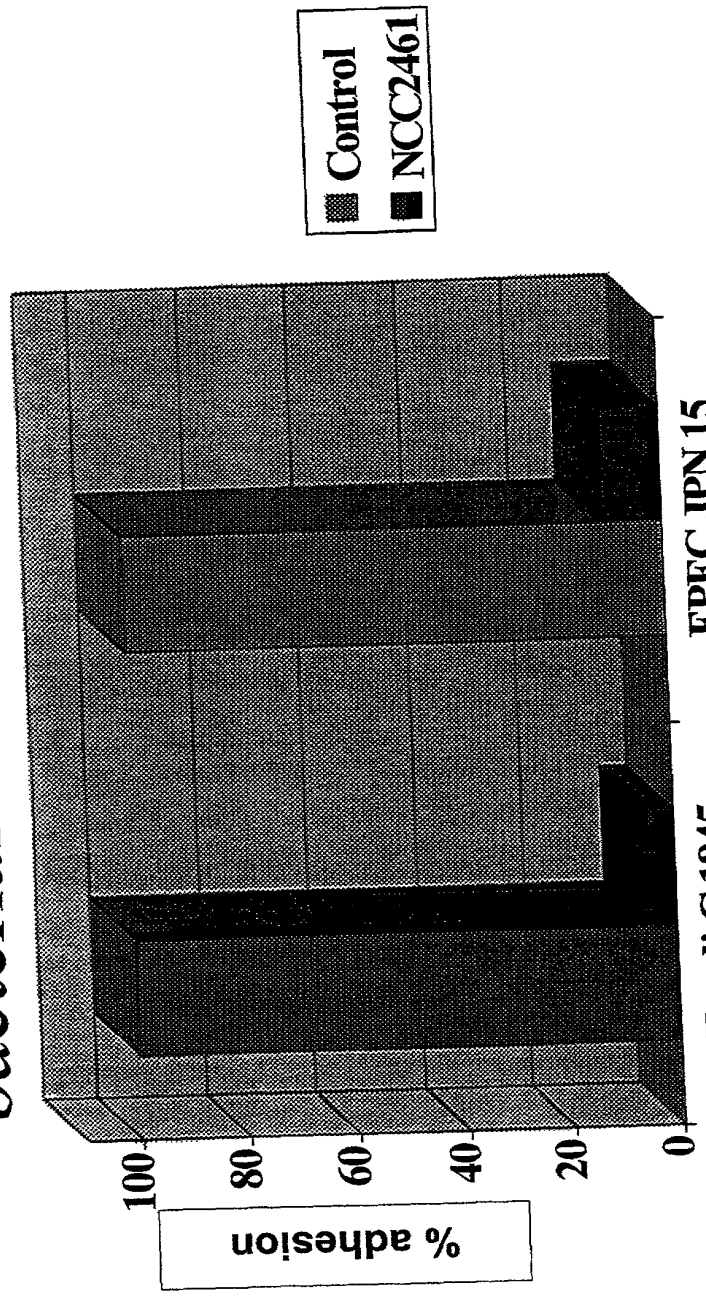
Inhibition of adhesion of enterovirulent
E. coli during the contact with the
bacterial culture NCC2461
Control
NCC2461
% adhesion
100
80
60
40
20
0
E. coli C 1845
EPEC JPN 15

FIG - 1

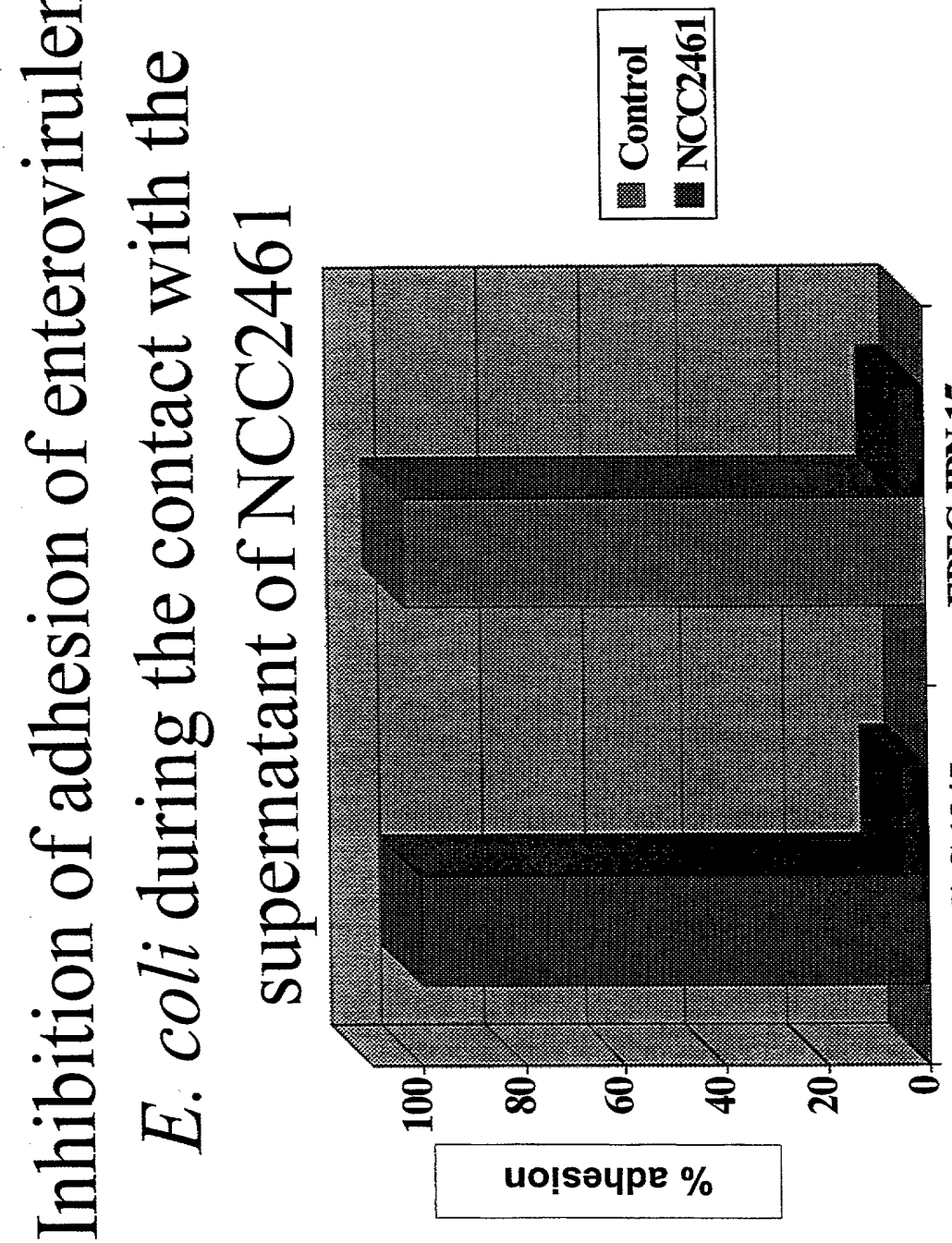
Inhibition of adhesion of enterovirulent
E. coli during the contact with the
supernatant of NCC2461
Control
NCC2461
% adhesion
100
80
60
40
20
0
E. coli C 1845
EPEC JPN 15

FIG - 2

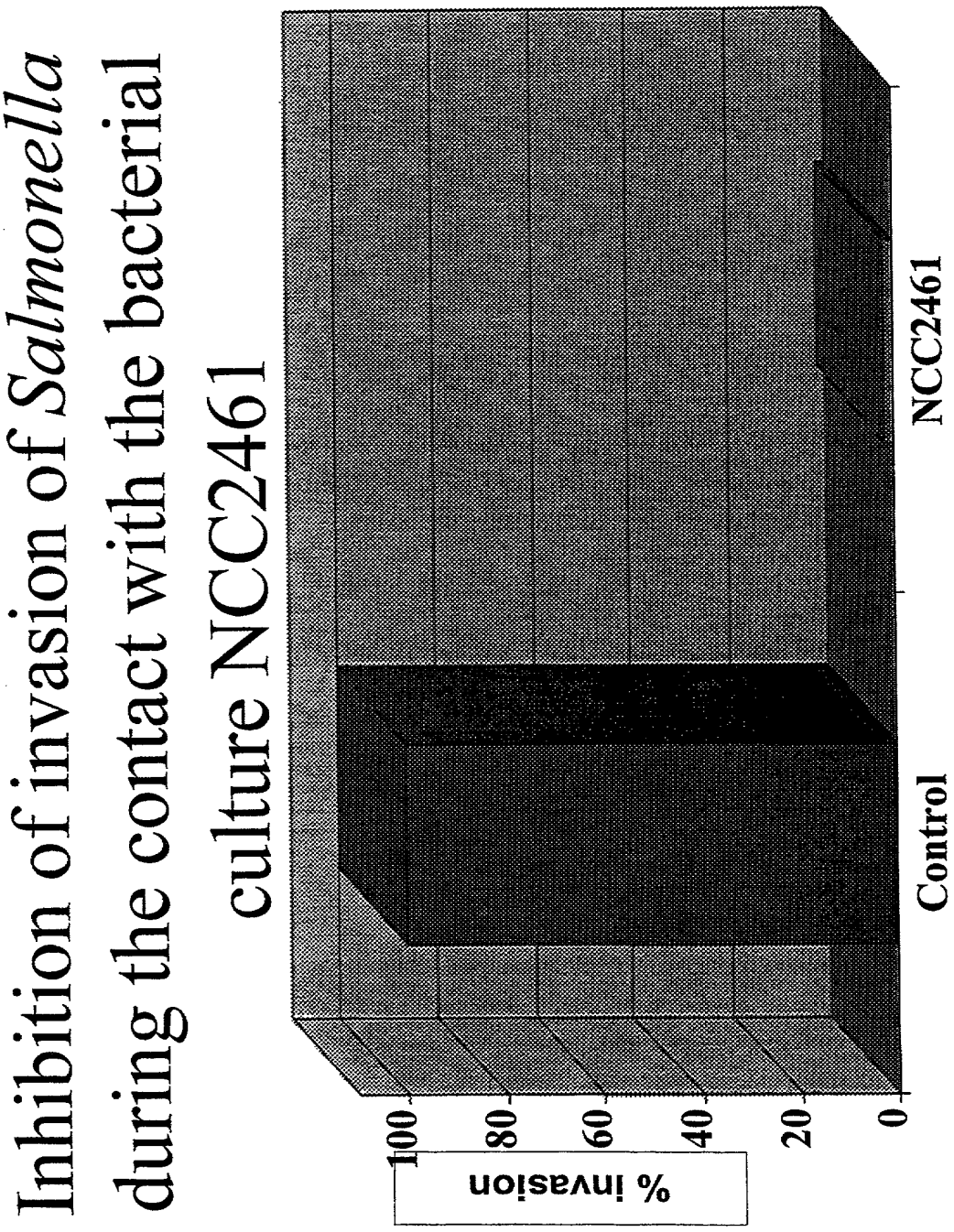
Inhibition of invasion of *Salmonella* during the contact with the bacterial culture NCC2461
% invasion
100
80
60
40
20
0
Control
NCC2461

FIG - 3

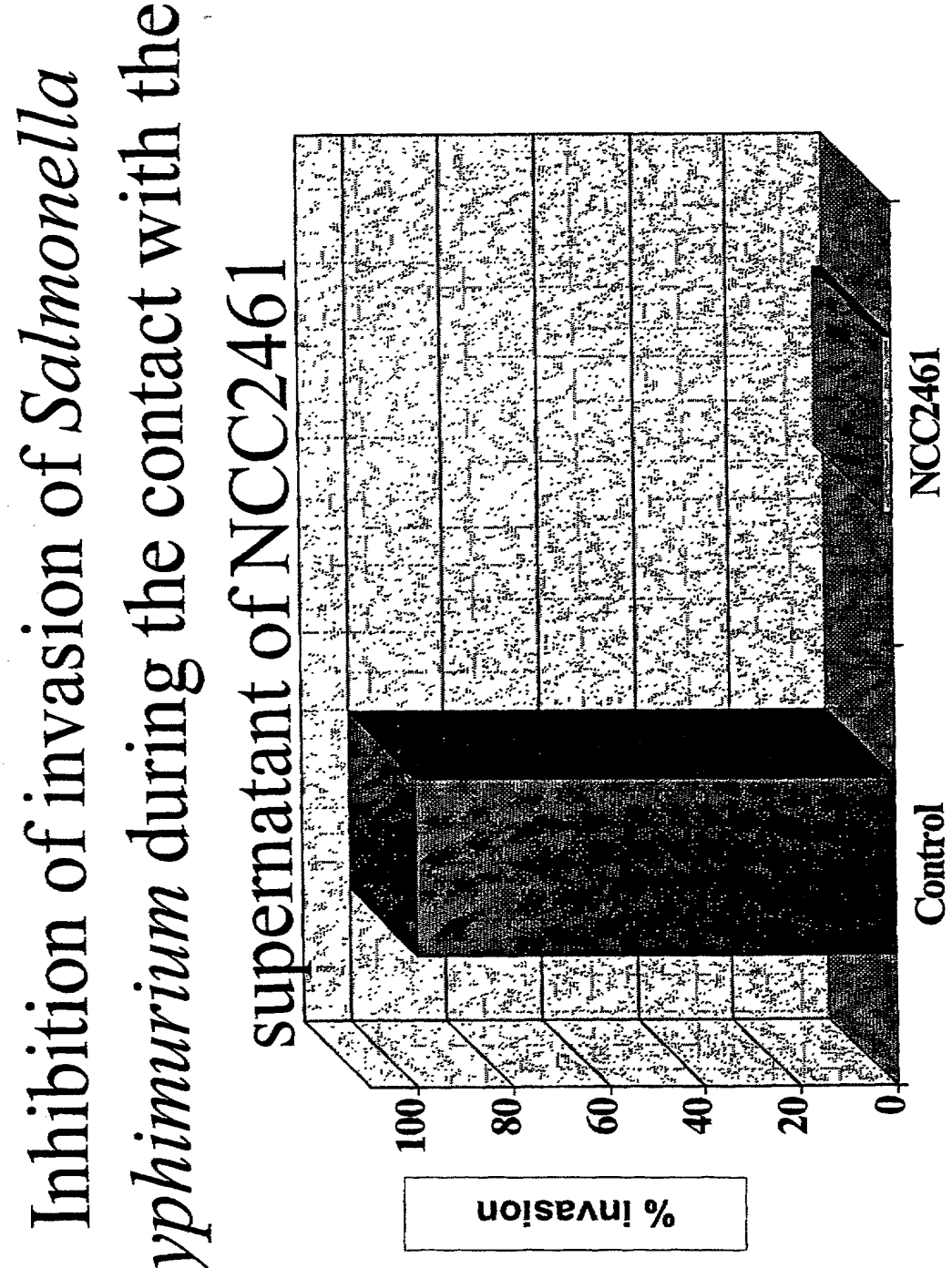
Inhibition of invasion of *Salmonella typhimurium* during the contact with the supematant of NCC2461
% invasion
100
80
60
40
20
0
Control
NCC2461

FIG - 4

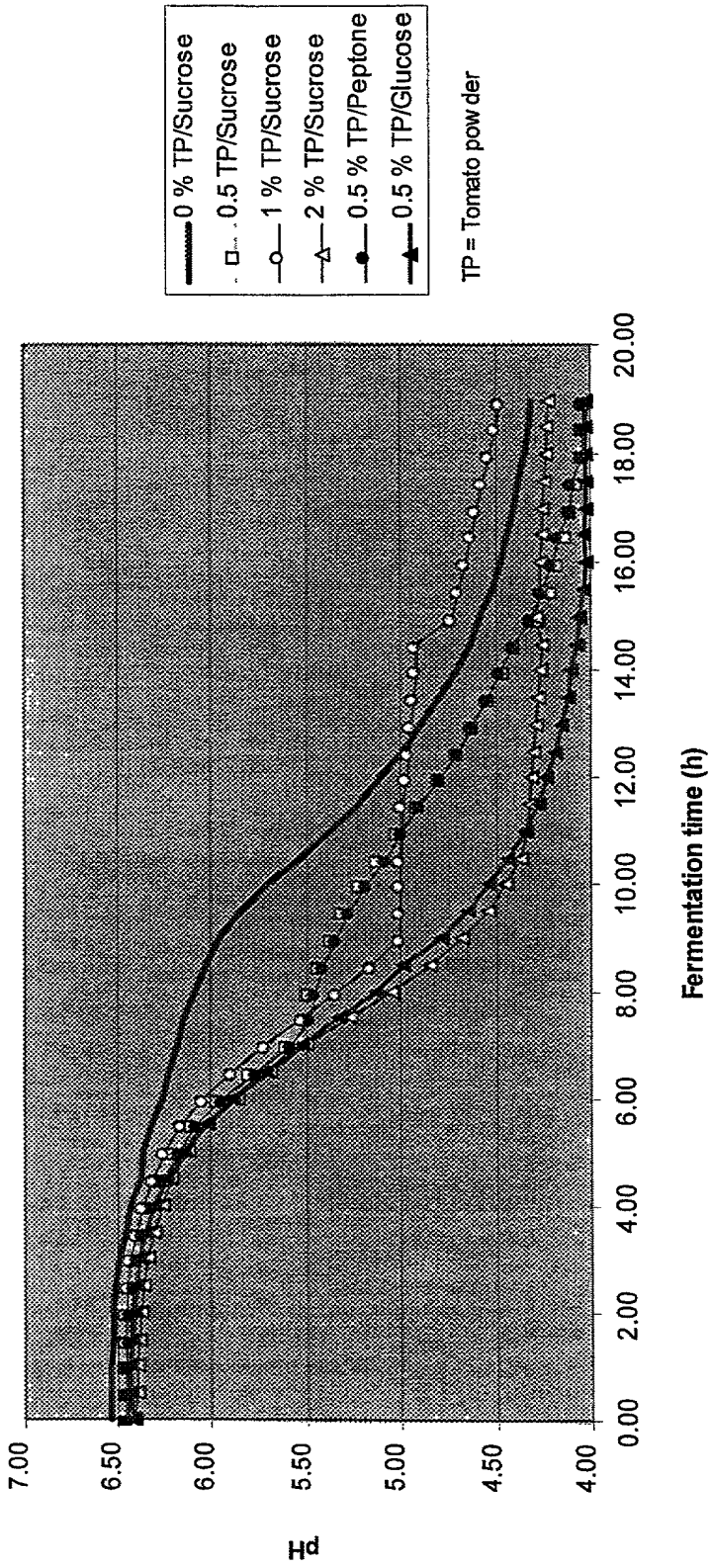
Acidification of L. casei ST11 in different growth media
0 % TP/Sucrose
0.5 TP/Sucrose
1 % TP/Sucrose
2 % TP/Sucrose
0.5 % TP/Peptone
0.5 % TP/Glucose
TP = Tomato pow der
pH
7.00
6.50
6.00
5.50
5.00
4.50
4.00
Fermentation time (h)
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00

FIG - 5

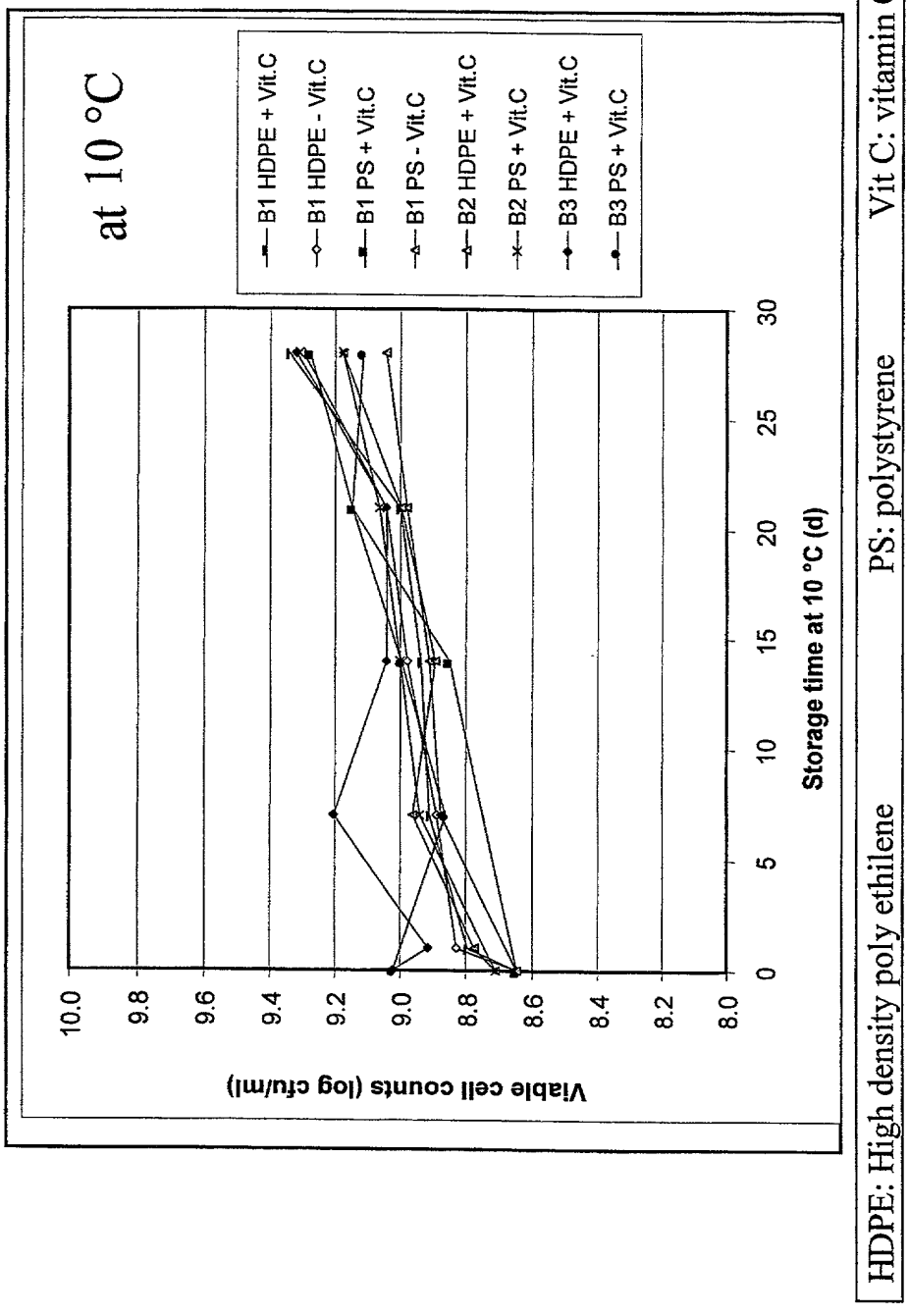
Survival in cereal drink
Survival of Lb. Casei ST11
at 10 °C
B1 HDPE + Vit.C
B1 HDPE - Vit.C
B1 PS + Vit.C
B1 PS - Vit.C
B2 HDPE + Vit.C
B2 PS + Vit.C
B3 HDPE + Vit.C
B3 PS + Vit.C
Viable cell counts (log cfu/ml)
10.0
9.8
9.6
9.4
9.2
9.0
8.8
8.6
8.4
8.2
8.0
0
5
10
15
20
25
30
Storage time at 10 °C (d)
HDPE: High density poly ethilene
PS: polystyrene
Vit C: vitamin C

FIG - 6

- Induction of cytokine mRNA by LAB in mouse macrophages

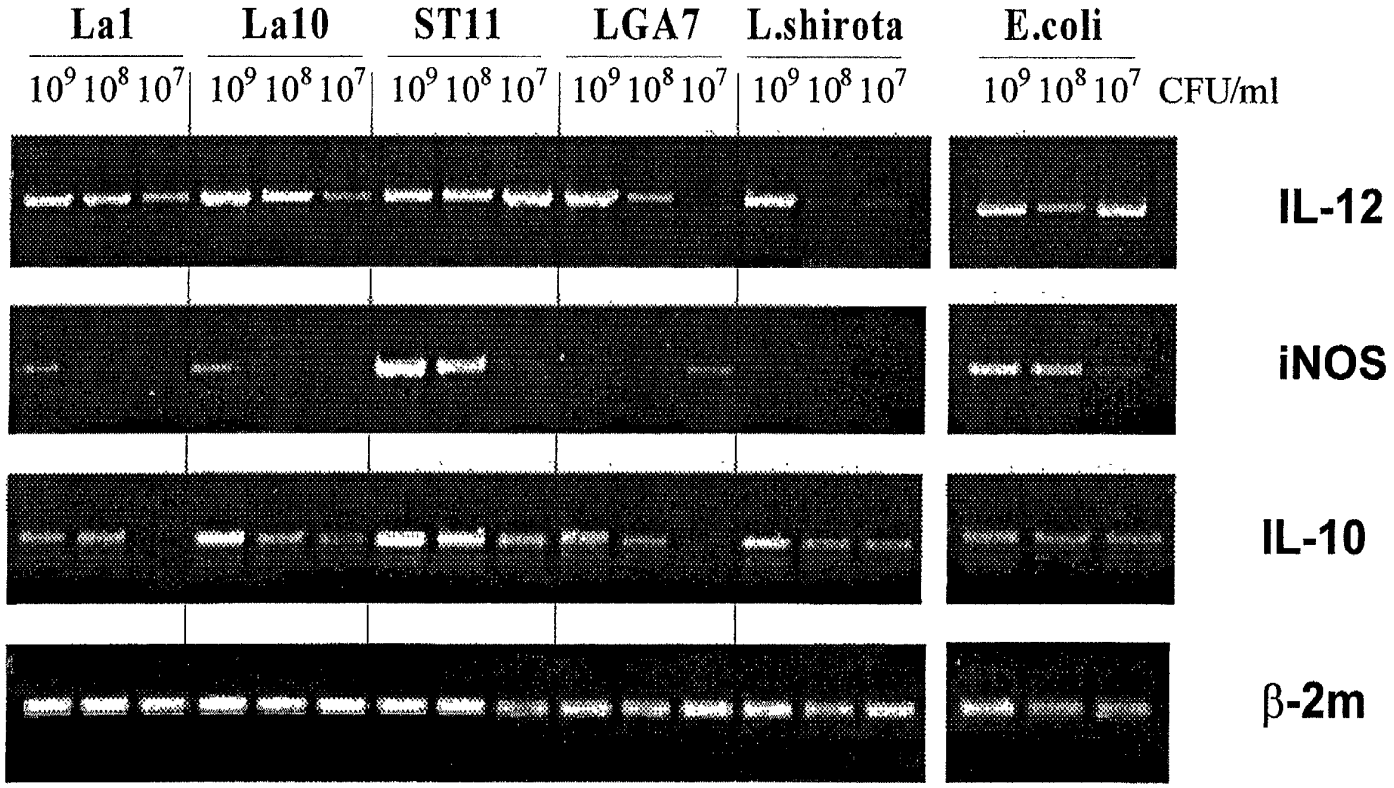

FIG - 7

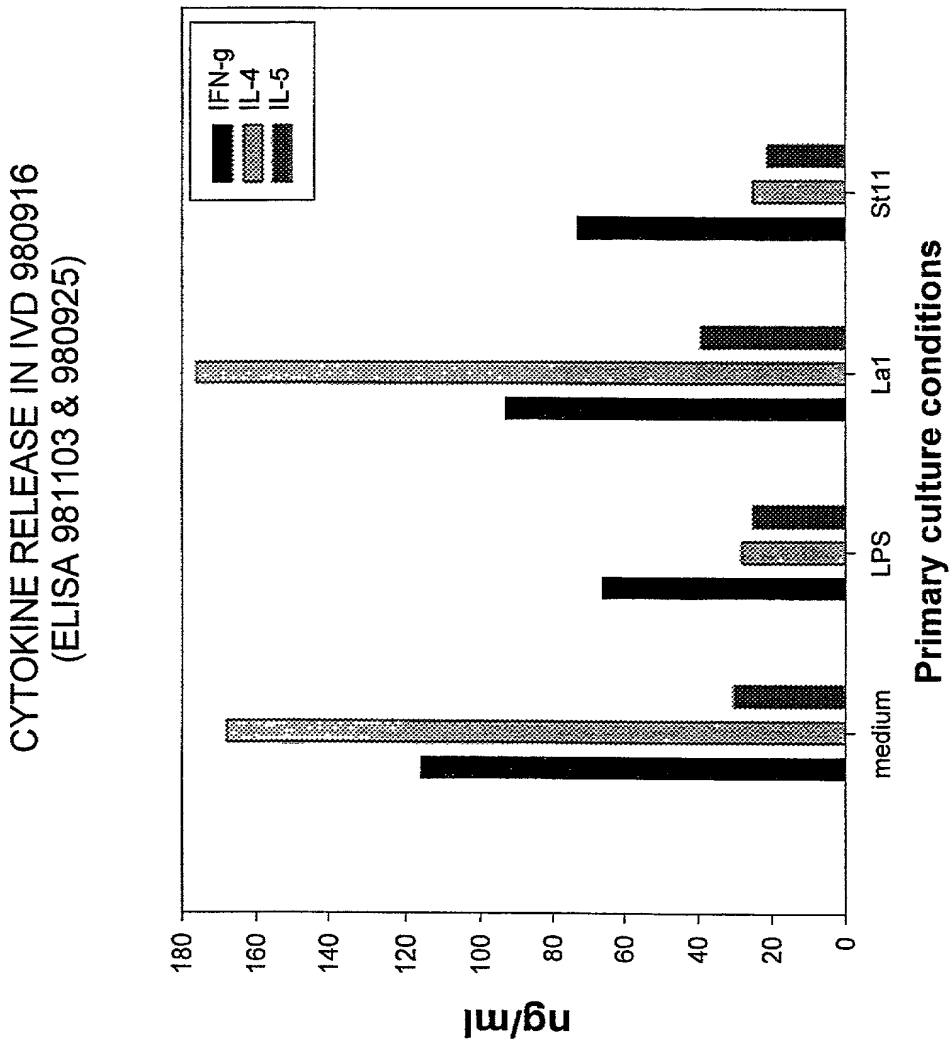
CYTOKINE RELEASE IN IVD 980916
(ELISA 981103 & 980925)
IFN-g
IL-4
IL-5
ng/ml
0
20
40
60
80
100
120
140
160
180
medium
LPS
La1
St11
Primary culture conditions

FIG - 8

Docket No.
112843-030

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
LACTOBACILLUS STRAINS PREVENTING DIARRHOEA PATHOGENIC BACTERIA

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on **2 March 2000** as United States Application No. or PCT International Application Number **PCT/EP00/01795**

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
PCT/EP00/01795	**WIPO**	**2 March 2000**	☐
(Number)	(Country)	(Day/Month/Year Filed)	
99104922.2	**Europe**	**11 March 1999**	☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Send Correspondence to: **Robert M. Barrett, Esq.**
Bell, Boyd & Lloyd LLC
P.O. Box 1135
Chicago, Illinois 60690-1135

Direct Telephone Calls to: *(name and telephone number)*
Robert M. Barrett (312) 807-4204

Full name of sole or first inventor **Jean-Richard Neeser**
Sole or first inventor's signature X Date October 26, 2001
Residence **Savigny, Switzerland** CH X
Citizenship **Switzerland**
Post Office Address **6, sentier de Courtaraye**
CH-1073 Savigny, Switzerland

Full name of second inventor, if any **Roberto Reniero**
Second inventor's signature X X Date October 29, 2001
Residence **Le Mont-Pelerin, Switzerland** CH X
Citizenship **Italy**
Post Office Address **24, chemin Paudille**
CH-1801 Le Mont-Pelerin, Switzerland

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Full name of third inventor, if any
Alain Servin

Third inventor's signature

Date

Residence
Chatenay-Malabry, France FRX

Citizenship
France

Post Office Address
UFR de Sc. Ph. Paris XI, Faculte de Pharmacie (Dep. Microb./Immunol.)

F-92296 Chatenay-Malabry, France

Full name of fourth inventor, if any

Fourth inventor's signature

Date

Residence

Citizenship

Post Office Address

Full name of fifth inventor, if any

Fifth inventor's signature

Date

Residence

Citizenship

Post Office Address

Full name of sixth inventor, if any

Sixth inventor's signature

Date

Residence

Citizenship

Post Office Address

United States Patent & Trademark Office

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